BASIC RESEARCH

Different effects of tirofiban and aspirin plus clopidogrel on myocardial no-reflow in a mini-swine model of acute myocardial infarction and reperfusion

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Heart 2006;**92**:1131–1137. doi: 10.1136/hrt.2005.077164

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Accepted
15 December 2005
Published Online First
30 December 2005

Objective: To compare the effects of an aspirin–clopidogrel combination with those of the specific glycoprotein IIb/IIIa inhibitor tirofiban on myocardial no-reflow, nitric oxide concentration and activity of nitric oxide synthase (NOS) isoforms in a mini-swine model of acute myocardial infarction and reperfusion.

Methods: Area of no-reflow was determined by both myocardial contrast echocardiography and pathological means in 40 mini-swine randomly assigned to five study groups: eight controls, eight pretreated with aspirin–clopidogrel combination for three days, eight given an intravenous infusion of tirofiban, eight treated with ischaemic preconditioning and eight sham operated. The acute myocardial infarction and reperfusion model was created with 3 h occlusion of the left anterior descending coronary artery followed by 1 h reperfusion.

Results: Compared with the control group, tirofiban significantly decreased the area of no-reflow assessed echocardiographically and pathologically, from 78.5% to 22.8% and 82.3% to 23.2%, respectively (both $p < 0.01$), and increased blood nitric oxide concentration ($p < 0.05$), enhanced constitutive NOS activity from 0.51 to 0.81 U/mg protein and mRNA expression from 0.47% to 0.66%, but decreased inducible NOS activity from 0.76 to 0.41 U/mg protein and mRNA expression from 0.54% to 0.39% in reflow myocardium (all $p < 0.05$–0.01). In contrast, the aspirin–clopidogrel combination did not significantly modify the above parameters (all $p > 0.05$) except for decreasing inducible NOS activity from 0.76 to 0.39 U/mg protein ($p < 0.01$) and mRNA expression from 0.54% to 0.40% ($p < 0.05$).

Conclusions: Tirofiban is very effective in attenuating myocardial no-reflow; in contrast, aspirin–clopidogrel combination is totally ineffective. These findings also support the concept that endothelial protection, apart from platelet inhibition, contributes to the beneficial effect of tirofiban on myocardial no-reflow.

The main goal of reperfusion therapy for acute myocardial infarction (AMI) is to restore both epicardial and microvascular blood flow to the ischaemic myocardium. Primary percutaneous coronary intervention, the preferred treatment for AMI, can achieve normal epicardial coronary flow. Studies have shown, however, that despite complete restoration of epicardial vessel blood flow, myocardial tissue perfusion evaluated with myocardial contrast echocardiography (MCE) remains incomplete, or even absent, known as slow flow or the no-reflow phenomenon,[1 2] which accounts for 37%[3] of patients with a first anterior AMI after receiving coronary reflow. No-reflow has been associated with severe myocardial injury, progressive left ventricular (LV) remodelling, congestive heart failure and poor prognosis.[4–6] Myocardial tissue perfusion is therefore now accepted as a target of reperfusion therapy for AMI.[7]

Specific platelet glycoprotein IIb/IIIa receptor inhibitors with powerful antiplatelet aggregation properties have been found to attenuate no-reflow.[8–10] The exact cause of this beneficial effect is, however, unclear. Aspirin and clopidogrel are also platelet inhibitors. The combination of aspirin and clopidogrel has been widely used to treat patients undergoing percutaneous coronary intervention and primary percutaneous coronary intervention after AMI to prevent subacute thrombosis, but its effect on myocardial no-reflow has not been evaluated. In addition, endothelial dysfunction, as it occurs during ischaemia and reperfusion,[11] predisposes to abnormal platelet–endothelial interaction with platelet activation and increased susceptibility to vasoconstriction,[12 13] and thus plays an important role in reperfusion injury. Notably, glycoprotein IIb/IIIa inhibition has been shown to have beneficial effects on endothelial function.[14 15] It is unknown, however, whether this beneficial effect of glycoprotein IIb/IIIa inhibition on myocardial no-reflow is also partly due to protection against endothelial dysfunction, which is characterised by decreased synthesis of endothelium-derived nitric oxide (NO).[16–18] NO synthase (NOS) isoforms contribute to the production of endogenous NO. In this study, we therefore used a mini-swine model of AMI and reperfusion developed in our laboratory to compare the

Abbreviations: AMI, acute myocardial infarction; CBV, coronary blood flow volume; cNOS, constitutive nitric oxide synthase; $\pm dp/dt_{max}$, maximum change rate of left ventricular pressure rise and fall; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IPC, ischaemic preconditioning; LAD, left anterior descending coronary artery; LV, left ventricular; MCE, myocardial contrast echocardiography; NO, nitric oxide; NOS, nitric oxide synthase; PCR, polymerase chain reaction; RESTORE, Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis; TARGET, Do Tirofiban And Reopro Give similar Efficacy outcome Trial

effects of aspirin–clopidogrel combination with those of the specific glycoprotein IIb/IIIa inhibitor tirofiban on myocardial no-reflow, the NO concentration and the activity of NOS isoforms.

METHODS

Animal preparation

The mini-swine (mean 30.3 (SD 3.0) kg) were anesthetised and ventilated with a respirator(SV 900; Siemens-Elema, Solna, Sweden). A middle thoracotomy was performed, and the heart was suspended in a pericardial cradle. The middle and distal portion of the left anterior descending coronary artery (LAD) was excised from surrounding tissue and was encircled by a suture. The two ends of the suture were threaded through a length of plastic tubing, forming a snare, which could be tightened to occlude the coronary artery. The right femoral artery and vein were cannulated for haemodynamic monitoring and contrast agent injection, respectively. An ultrasonic flow probe was placed proximal to the site of occlusion. The probe was connected to a flowmeter (Nihon Kohden Corporation) for digital measurement of LAD flow.

Experimental protocol

Forty animals were randomly assigned to five study groups according to a previous study[8]: eight controls, eight treated with tirofiban, eight pretreated with combined aspirin–clopidogrel, eight treated with ischaemic preconditioning (IPC), and eight sham operated. In aspirin–clopidogrel combination pretreated animals, a 300 mg loading dose, followed by 75 mg/day of clopidogrel (donated by Sanofi-Synthelabo Minsheng Pharmaceutical Company, Hangzhou, China) plus 10 mg/kg/day of aspirin (donated by AstraZeneca Pharmaceutical Company), was given three days before the protocol so that platelet inhibition reached a steady state. In the tirofiban-treated animals, the dose of tirofiban was 1.5-fold higher than that in the RESTORE (Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis) and TARGET (Do Tirofiban And Reopro Give similar Efficacy outcome Trial) studies[19 20] based on body surface area.[21] Thus, tirofiban (Wuhan Yuanda Pharma Co, Wuhan, China) was given as a 15 μg/kg intravenous bolus, followed by an infusion administered at a rate of 0.3 μg/kg/min from 30 min before occlusion to the end of the protocol, as the aspirin–clopidogrel combination was given before occlusion. Control animals received the same amount of saline intravenously. In IPC animals, the LAD was occluded for 10 min, followed by 5 min of reperfusion, the procedure being repeated three times before the protocol. The AMI and reperfusion model was created with 3 h of LAD occlusion followed by 60 min of reperfusion, as previously described for animal studies of the no-reflow phenomenon.[8 22] In the sham-operated animals, the LAD was only encircled by a suture, but not occluded. Data were collected at baseline, at the end of 3 h of LAD occlusion, and at 60 min of reperfusion.

Assessment of platelet function

Blood samples were collected in citrate-anticoagulated plastic tubes at two time points: before treatment (at baseline) and after treatment (immediately before LAD occlusion). Platelet function was assayed and evaluated by blinded staff. Platelet-rich and platelet-poor plasma samples were prepared by centrifugation at 1000 rpm for 3 min and 3000 rpm for 10 min, respectively, at room temperature. After calibration with platelet-rich and platelet-poor plasma samples, we measured ex vivo platelet aggregation in response to 20 μmol/l ADP in platelet-rich plasma by using optical light-transmission aggregometry (Chrono-log Corporation, Havertown, Pennsylvania, USA).

Haemodynamic function

At baseline, at the end of 3 h of LAD occlusion and at 60 min of reperfusion, we measured heart rate, LV systolic pressure, LV end diastolic pressure, maximum change rate of LV pressure rise and fall ($\pm dp/dt_{max}$) and cardiac output. Coronary blood flow volume (CBV), which reflects myocardial tissue perfusion indirectly, was also measured at baseline (before occlusion), immediately after release of occlusion (3 h), and at 60 min of reperfusion.

MCE evaluation

Echocardiography was performed with an HP 5500 machine (Philips Ultrasound). The transducer was fixed in position to obtain the same short-axis images of the left ventricle at the mid-papillary muscle level. A warm-water bath acted as an acoustic interface between the heart and the transducer. A bolus of 0.05 ml/kg of Sonovue (Bracco Inc, Geneva, Switzerland) was injected intravenously as a slow bolus during 30 s followed by 5 ml saline flush. Data were collected at baseline, at the end of 3 h of LAD occlusion and at 60 min of reperfusion. For each MCE, end diastolic images were acquired at a pulsing interval of four cardiac cycles during contrast injection to allow complete beam replenishment and demarcation between perfused and non-perfused tissue. The myocardial ligation area and the area of no-reflow were identified as the region of non-opacified myocardium by MCE at 3 h of LAD occlusion and at 60 min of reperfusion, respectively. Ligation area, area of no-reflow and LV wall area were traced and measured. Ligation area was expressed as a percentage of the LV wall area and area of no-reflow was expressed as a percentage of the ligation area.

Histopathological evaluation

After completion of the experimental protocol, the area of no-reflow was delineated by intra-atrial injection of 1 ml/kg of the fluorescent dye thioflavin S (Sigma Chemical Co). Then the LAD was reoccluded, and Evans blue dye was injected into the left atrium to determine ligation area. The swine was then killed and the heart explanted. Five LV slices were cut parallel to the atrioventricular groove and incubated in a 1% solution of triphenyltetrazolium chloride for 15 min at 37℃. Under an ultraviolet light in a dark room, the areas not perfused by thioflavin S were identified. Ligation area was defined as the region unstained by Evans blue. The area of no-reflow was defined as the non-fluorescent area within the ligation area. Regions that were not stained red were considered to be the necrosis area. The outlines of the LV wall area, ligation area, area of no-reflow and necrosis area were calculated. Ligation area was expressed as a percentage of the LV wall area, and area of no-reflow and necrosis area were expressed as a percentage of the ligation area. Samples were then taken from the myocardium in the three regions, washed thoroughly with saline, and snap frozen in liquid nitrogen.

Measurement of NO

NO in vein blood samples was evaluated at baseline, at 5 min and 3 h of LAD occlusion, and at 5 min and 60 min of reperfusion. The serum was centrifuged at 3000 rpm for 10 min to obtain the supernatant, which was stored at −70℃ before analysis. The final products of NO in vivo are nitrite and nitrate. Total nitrite plus nitrate concentration was measured with use of the Griess reagent as described previously.[23 24] In brief, the supernatant was incubated with nitrate reductase in 20 mM Tris buffer (pH 7.5). The reaction was terminated after 60 min at 37℃, followed by the addition of Griess reagent, which converts nitrite to a deep purple azo compound; photometric measurement of the absorbance at 540 nm of this azochromophore accurately

Table 1 Effects of tirofiban and aspirin–clopidogrel combination on maximum aggregation rate

	Maximum aggregation rate (%)	
Group	Baseline	After treatment
Control (n = 8)	43.2 (4.38)	44.5 (6.51)
Tirofiban (n = 8)	46.8 (7.15)	12.9 (3.63)*†
Combination (n = 8)	45.7 (6.17)	14.3 (5.58)*†
IPC (n = 8)	44.7 (5.81)	45.1 (7.02)

Data are expressed as the mean (SD).
*p<0.01 *v* baseline; †p<0.01 *v* control.
IPC, ischaemic preconditioning.

determines the nitrite concentration (sodium nitrate is used as a standard). NO concentration was then calculated on the basis of a standard curve for $NaNO_2$ and expressed in micromoles per litre.

NOS activity assay

Constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) activity were determined by a NOS assay kit (TNOS/iNOS, cat no A014-1, coefficient of variation < 1.8%; Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). The NOS activity assay kit is based on the biochemical conversion of L-arginine to L-citrulline by NOS.[25 26] Measuring NOS activity by monitoring the conversion to NO is a standard assay for NOS activity in both crude and purified enzyme preparations. The NO that is produced can react with the nucleophilic compound to produce a coloured chemical compound; photometric measurement of the absorbance at 530 nm determines NOS activity. According to whether it is Ca^{2+} dependent, NOS can be divided into two types: cNOS (Ca^{2+} dependent) and iNOS (Ca^{2+} independent). The tissue lysates were sonicated with lysis buffer for 5 s twice on ice and centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant was collected and stored at −70°C before analysis. Protein concentration was determined by the Bradford method.[27] The supernatant was incubated with 0.6 ml reaction buffer (5 mmol/l $MgCl_2$, 250 mmol/l L-valine, 0.2 mol/l phosphate-buffered saline, 480 μmol/l oxyhaemoglobin and 30 mmol/l NADPH), combined with the addition of 1 mmol/l $CaCl_2$ or inhibitor of cNOS (6 mmol/l EGTA). The reaction was terminated after 15 min at 37°C with 10 mmol/l EDTA and 10 mmol/l HEPES buffer. The formation of a coloured chemical compound was photometrically measured by a spectrophotometer (Sorvall, Fresno, California, USA) at 530 nm. Total NOS and iNOS activity was then measured based on the Lambert–Beer Law. cNOS activity was calculated by subtracting iNOS from total NOS. NOS activity was expressed as units per milligram of protein.

Reverse transcription polymerase chain reaction for NOS isoform mRNA

Total RNA was extracted from an aliquot of the fresh frozen tissue. Total RNA from each sample was reverse transcribed and specific genes were further amplified by polymerase chain reaction (PCR) with 10% of the reverse transcription product each time. The message level of GAPDH was used as an internal control to assess the quality and quantity of RNA extraction and efficiency of the reverse transcription PCR. The primers for GAPDH, iNOS and endothelial NOS (eNOS) were designed with Primer Express software (Applied Biosystems, Foster City, California, USA) and synthesised by Beijing Dingguo Bioengineering Institute (Beijing, China). Their sequences for amplification were $_{(+)}$ 5′-CCA TGG AGA AGG CTG GG-3′ $_{(-)}$ 5′-CAA AGT TGT CAT GGA TGA CC-3′ for GAPDH, $_{(+)}$5′-CTC TTC GAA ATC CCT CCT GAC- 3′ $_{(-)}$ 5′-GAC ATT GAT CTC CAC GAC ACG- 3′ for iNOS and $_{(+)}$5′-CTG CAT GAC ATT GAG AGC AAA- 3′ $_{(-)}$ 5′-AAT GTC CTC GTG ATA GCG TTG- 3′ for eNOS. Ten microlitres of PCR product was electrophoresed on a 1% agarose gel with ethidium bromide staining for visualisation. The gels were then photographed and quantified by a gel documentation system. For all RNA samples, the density of an individual mRNA band was divided by that of the GAPDH mRNA band to correct for differences in RNA loading or transfer.

Statistical methods

Data are expressed as mean (SD). Data from all stages were compared by repeated measures analysis of variance followed by the Student–Newman–Keuls test for multiple comparison. Ligation area, area of no-reflow and necrosis area were compared between groups by one-way analysis of variance followed by Dunnett's test for multiple comparison with control. A value of $p < 0.05$ (two sided) was considered significant.

RESULTS

Platelet function

Compared with the baseline rate, maximum aggregation rate was significantly reduced after treatment in both the aspirin–clopidogrel combination and tirofiban groups (both $p < 0.01$). Maximum aggregation rate was also significantly reduced in both the aspirin–clopidogrel combination and tirofiban groups (both $p < 0.01$) and did not change in the IPC group ($p > 0.05$) compared with that in control. Maximum aggregation rate did not differ significantly between the aspirin–clopidogrel combination and tirofiban groups ($p > 0.05$) (table 1).

No-reflow and infarct size

In the control group, the coronary ligation area was similar on MCE in vivo and on pathological evaluation ($p > 0.05$). The area of no-reflow was also similar (8.5% and 82.3% by the two methods, respectively), with final necrosis area reaching 99% of ligation area. Ligation area did not differ significantly by MCE and pathological evaluation between the three treated and control groups. Compared with the control group, in tirofiban-treated and IPC groups the area of no-reflow by both assessment methods was significantly decreased to 22.8–23.2% and 16.4–17.5% (all $p < 0.01$), with the final necrosis area significantly reduced to 89.2% and 78.4% of the ligation area (both $p < 0.05$). In contrast, both the area of no-reflow and the necrosis area in the aspirin–clopidogrel combination group were not significantly different from those in the control group (both $p > 0.05$) and were significantly higher than those in the tirofiban group (both $p < 0.01$) (table 2).

Coronary blood flow volume

In the control group, CBV was significantly reduced to 45.8% and 50.6% of the baseline immediately after release of occlusion (3 h) and at 60 min of reperfusion, respectively (both $p < 0.01$). In the tirofiban-treated and IPC groups, CBV was also significantly reduced at these two time points compared with the baseline (tirofiban, 72.5% and 73.2%; IPC, 74.1% and 74.5% of the baseline, respectively, $p < 0.05$), but was significantly higher than CBV in the control group (both $p < 0.01$). In contrast, CBV in the aspirin–clopidogrel combination group was not significantly different from that in the control group ($p > 0.05$) and was significantly lower than that in the tirofiban group (both $p < 0.01$) (fig 1).

Haemodynamic function

Haemodynamic parameters did not differ significantly between the four groups at baseline (all $p > 0.05$). In the control group, LV systolic pressure, $\pm dp/dt_{max}$ and cardiac

Table 2 Effects of tirofiban and aspirin–clopidogrel combination on ANR and NA

	MCE		Histopathological evaluation		
Group	LA (%)	ANR (%)	LA (%)	ANR (%)	NA (%)
Control (n = 8)	22.4 (3.02)	78.5 (4.35)	23.5 (1.98)	82.3 (1.90)	98.5 (1.35)
Tirofiban (n = 8)	22.7 (2.98)	22.8 (4.21)**	23.6 (3.56)	23.2 (1.86)**	89.2 (2.77)*
Combination (n = 8)	22.9 (2.68)	77.9 (5.07)	23.9 (4.11)	81.75 (3.21)	98.15 (0.89)
IPC (n = 8)	22.3 (2.09)	16.4 (2.24)**	23.8 (3.55)	17.5 (2.87)**	78.4 (1.62)*

Data are expressed as the mean (SD).
*p<0.05, †p<0.01 *v* control group.
ANR, area of no-reflow; IPC, ischaemic preconditioning; LA, ligation area; MCE, myocardial contrast echocardiography; NA, necrosis area.

output fell significantly ($p < 0.05$–0.01), whereas LV end diastolic pressure increased significantly at the end of 3 h of LAD occlusion ($p < 0.01$) with $\pm dp/dt_{max}$ falling significantly further (both $p < 0.05$) at 60 min of reperfusion relative to that at LAD occlusion. In the three treated groups, the changes of LV systolic pressure, $\pm dp/dt_{max}$, cardiac output and LV end diastolic pressure were the same as those in the control group after coronary occlusion and reperfusion, although $\pm dp/dt_{max}$, cardiac output and LV end diastolic pressure recovered significantly at 60 min of reperfusion only in the tirofiban-treated and IPC groups (table 3).

NO in blood samples

In the control group, NO in blood was significantly increased at 5 min of AMI ($p < 0.05$) but decreased at 3 h of AMI, 5 min of reperfusion and 1 h of reperfusion (all $p < 0.01$) relative to the baseline. Compared with that at 3 h of AMI, the NO concentration at 5 min and at 1 h of reperfusion was significantly increased (both $p < 0.01$). In the tirofiban-treated and IPC groups, NO concentration was significantly higher than that in the control group ($p < 0.05$). In contrast, NO did not differ significantly at 5 min of AMI and 3 h of AMI between the aspirin–clopidogrel combination and control groups but was significantly lower at 5 min and at 1 h of reperfusion in the aspirin–clopidogrel combination group than in the control group ($p < 0.05$) (fig 2).

NOS isoform activity in the myocardium

In the control and in the three treated groups, cNOS activity in the reflow and no-reflow myocardium was significantly lower and iNOS activity was significantly higher than in normal myocardium (all $p < 0.01$). cNOS activity decreased further and iNOS activity increased in no-reflow myocardium relative to reflow myocardium (all $p < 0.01$). In the tirofiban-treated and IPC groups, cNOS activity in the reflow myocardium was significantly higher than that in the control group ($p < 0.01$). In contrast, cNOS activity did not differ significantly between the aspirin–clopidogrel combination and control groups ($p > 0.05$). In the three treated groups, the level of iNOS activity in the reflow myocardium was significantly lower than that in the control group ($p < 0.01$) (fig 3).

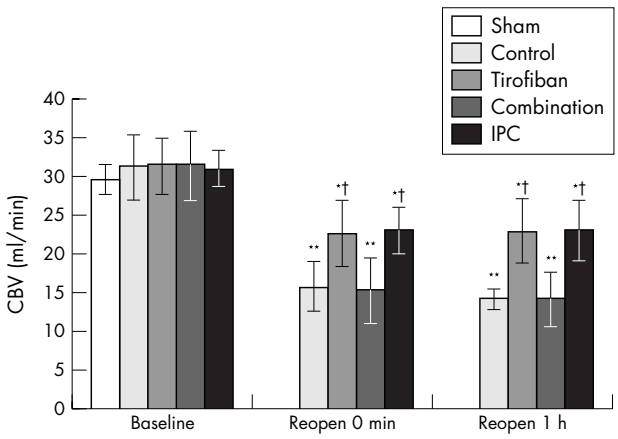

Figure 1 Coronary blood flow volume (CBV) in five groups at baseline, at release of occlusion and 1 h after release of occlusion. Mean (SD), n = 8. *$p < 0.05$, **$p < 0.001$ *v* baseline; †$p < 0.01$ *v* control group. IPC, ischaemic preconditioning.

NOS isoform mRNA in the myocardium

In the control and the three treated groups, eNOS mRNA was significantly lower and iNOS mRNA was significantly higher in the reflow and no-reflow myocardium than in normal myocardium ($p < 0.01$). In no-reflow myocardium eNOS mRNA decreased and iNOS mRNA increased further in comparison with those in reflow myocardium ($p < 0.01$). In the tirofiban-treated and IPC groups, eNOS mRNA in the reflow myocardium was significantly higher than that in the control group ($p < 0.01$). In contrast, eNOS mRNA did not differ significantly between the aspirin–clopidogrel combination and control groups ($p > 0.05$). In the three treated groups, the level of iNOS mRNA in the reflow myocardium was significantly lower than that in the control group ($p < 0.05$) (fig 4).

DISCUSSION

To improve the clinical outcome of coronary stenting, several platelet-inhibiting agents have been developed. These include clopidogrel, which blocks the platelet ADP receptor, and the glycoprotein IIb/IIIa receptor inhibitors abciximab and tirofiban, which compete with fibrinogen binding to its platelet receptor. Although glycoprotein IIb/IIIa receptor inhibitors, together with adjuvant treatment with an aspirin and clopidogrel combination, is used widely in interventional cardiology, their differential effects on myocardial no-reflow have not been compared.

It is noteworthy that the glycoprotein IIb/IIIa receptor inhibitor tirofiban can decrease the area of no-reflow and improve CBV, whereas the aspirin and clopidogrel combination was totally ineffective in the present study. Previous studies[8 28] have provided evidence of the beneficial effect of glycoprotein IIb/IIIa receptor inhibitors on the microvasculature. Our data are the first to establish directly that aspirin and clopidogrel in combination exert no favourable effect on capillary perfusion after restoration of flow in epicardial vessels.

The proposed mechanism of the no-reflow phenomenon is multifactorial. Although it has been suggested that plugging of capillaries by leucocytes and platelet activation contribute to no-reflow,[29 30] these blood cell elements are not necessary for the development of this phenomenon, because no-reflow has been observed in buffer-perfused hearts as well.[31 32] As shown in animal models of coronary artery occlusion and reperfusion, localised endothelial swelling and protrusions

Table 3 Effect of tirofiban and aspirin–clopidogrel combination on haemodynamic function

Group	HR (beats/min)	LVSP (mm Hg)	LVEDP (mm Hg)	$+dp/dt_{max}$ (mm Hg/s)	$-dp/dt_{max}$ (mm Hg/s)	CO (l/min)
Sham (n = 8)						
Baseline	110 (4)	115 (5)	3.9 (2.0)	2900 (541)	2612 (112)	2.67 (0.12)
Control (n = 8)						
Baseline	109 (5)	115 (4)	4.0 (1.5)	2850 (547)	2538 (207)	2.58 (0.36)
Ischaemia 3 h	107 (6)	100 (4)**	7.1 (2.0)**	2475 (468)*	2275 (191)*	1.26 (0.29)**
Reperfusion 1 h	108 (6)	109 (3)**‡	6.1 (1.6)**	2287 (551)**‡	2112 (242)**‡	1.34 (0.25)**
Tirofiban (n = 8)						
Baseline	109 (8)	116 (7)	3.9 (2.6)	2889 (453)	2568 (189)	2.49 (0.37)
Ischaemia 3 h	108 (9)	101 (5)**	6.7 (2.3)**	2456 (436)**	2164 (159)**	1.22 (0.26)**
Reperfusion 1 h	107 (9)	111 (2)*†‡	4.3 (1.9)**†‡	2759 (492)*†‡	2419 (183)*†‡	1.94 (0.31)**†‡
Combination (n = 8)						
Baseline	108 (8)	115 (6)	3.9 (2.5)	2775 (437)	2589 (208)	2.54 (0.47)
Ischaemia 3 h	107 (9)	102 (6)**	6.9 (1.9)**	2425 (306)*	2290 (189)*	1.27 (0.26)**
Reperfusion 1 h	109 (9)	108 (3)**‡	6.1 (2.1)**	2268 (437)**‡	2098 (211)**‡	1.39 (0.28)**
IPC (n = 8)						
Baseline	110 (6)	115 (3)	3.8 (2.1)	2950 (444)	2450 (151)	2.50 (0.30)
Ischaemia 3 h	109 (5)	100 (6)**	6.6 (1.5)**	2550 (469)**	2200 (169)**	1.23 (0.17)**
Reperfusion 1 h	109 (5)	111 (4)*†‡	4.8 (2.0)*†‡	2788 (470)*†‡	2337 (130)*†‡	1.91 (0.28)*†‡

Data are expressed as the mean (SD).
*p<0.05, **p<0.01 *v* baseline; †p<0.05 *v* control; ‡p<0.05 *v* ischaemia 3 h.
CO, cardiac output; $\pm dp/dt_{max}$, maximum change rate of left ventricular pressure rise and fall; HR, heart rate; IPC ischaemic preconditioning; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure.

are predominantly confined to the capillary bed.[32] Furthermore, abundant evidence shows that IPC prevents endothelial dysfunction after ischaemia and reperfusion,[33 34] whereas our study showed that IPC can decrease the area of no-reflow and improve CBV, indicating that endothelium has an important role in tissue-level perfusion. The mechanism by which glycoprotein IIb/IIIa inhibition is beneficial in the prevention of no-reflow is thought to be linked to distal vasoconstriction induced by activated platelets and platelet aggregates in the animal model of mechanical non-thrombotic coronary artery occlusion.[8 35] The present study showed that the degree of platelet aggregation (~72% of baseline values) reached by the tirofiban dose we used is not significantly different from that (~69% of baseline values) obtained by the combination of aspirin and clopidogrel. Interestingly, although the aspirin and clopidogrel combination, which is considered to be weaker than tirofiban, significantly induced platelet inhibition, it was totally ineffective in improving tissue-level reperfusion, indicating that the effect of tirofiban on myocardial no-reflow may be linked to a mechanism beyond platelet inhibition.

What is the possible mechanism for this different effect of antiplatelet treatment with tirofiban and aspirin–clopidogrel combination on no-reflow? Previous studies showed that the glycoprotein IIb/IIIa receptor is not only the final site of platelet aggregation, but also of paramount importance in mediating firm attachment of platelets to the vascular endothelium.[36 37] During the adhesion process, platelets are activated and release a variety of substances to mediate endothelial function (especially leucocyte adhesion and infiltration[38 39]), thus further exacerbating no-reflow.[40] Glycoprotein IIb/IIIa inhibitors can inhibit platelet-induced endothelial injury by binding to the glycoprotein IIb/IIIa receptor, whereas the aspirin–clopidogrel combination has no similar effect. The possible mechanism of glycoprotein IIb/IIIa inhibition in preventing no-reflow therefore is preventing firm attachment of platelets to the vascular endothelium and then protecting the endothelium from injury.

Our study also provides important evidence for endothelial protection by tirofiban after ischaemia and reperfusion. Endothelial dysfunction can be characterised by decreased synthesis of endothelium-derived NO.[16–18] NOS isoforms are the enzymes responsible for NO generation. NOS isoforms are divided into two categories according to calmodulin and calcium: cNOS (including neuronal NOS (type 1) and eNOS (type 3)) and iNOS (type 2). cNOS and iNOS are produced mainly by the vascular endothelium and macrophages, respectively.[41] Data show that NO synthesised by cNOS has a cardioprotective role, whereas high concentrations of NO synthesised by iNOS rapidly interact with oxygen to yield the

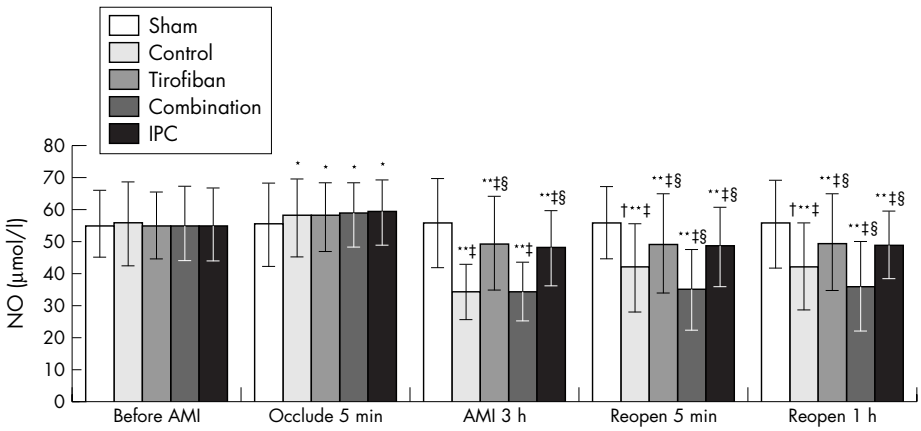

Figure 2 Variation of nitric oxide (NO) in serum at every time point. *p < 0.05, **p < 0.01 *v* before acute myocardial infarction (AMI); †p < 0.01 *v* AMI 3 h; ‡p < 0.01 *v* sham group; §p < 0.05 *v* control group. Mean (SD), n = 8 per group. IPC, ischaemic preconditioning.

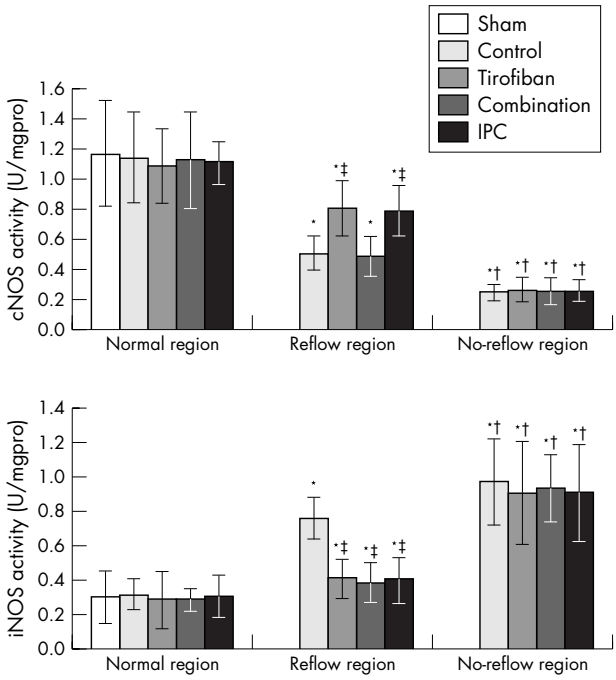

Figure 3 Variation of constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) activity in the myocardium. Mean (SD), n = 8 per group. *p < 0.01 *v* normal region; †p < 0.01 *v* reflow region; ‡p < 0.01 *v* control group. IPC, ischaemic preconditioning.

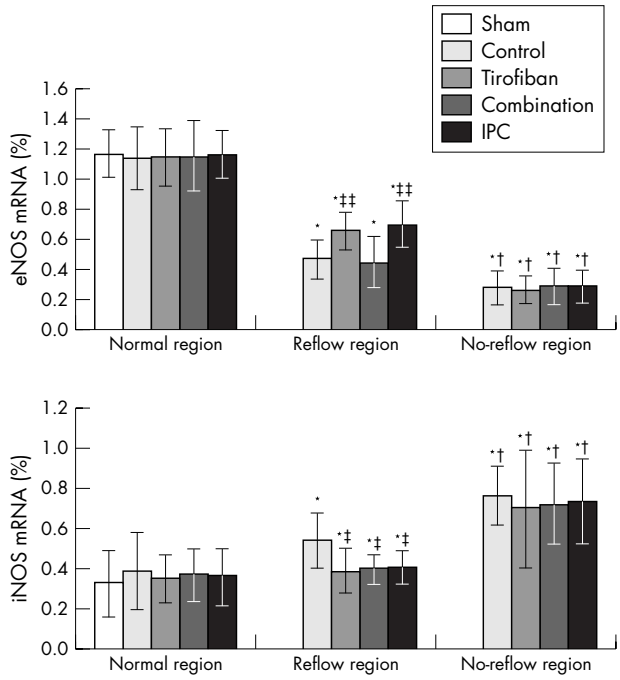

Figure 4 Variation of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) mRNA in the myocardium. Mean (SD), n = 8 per group. *p < 0.01 *v* normal region; †p < 0.01 *v* reflow region; ‡p < 0.05, ‡‡p < 0.01 *v* control group. IPC, ischaemic preconditioning.

potent oxidant peroxynitrite ($ONOO^-$) and subsequently induce and exacerbate myocardial ischaemia reperfusion injury.[42] The present study showed that tirofiban can increase blood NO concentration, cNOS activity and eNOS mRNA expression but decrease iNOS activity and mRNA expression in reflow myocardium, whereas the aspirin–clopidogrel combination can only decrease iNOS activity and mRNA expression in reflow myocardium. This suggests that tirofiban has a protection effect on endothelial function and the inflammatory response after reperfusion to some extent, but that aspirin and clopidogrel in combination can only attenuate the inflammatory response after reperfusion. Correspondingly, clinical study has shown that glycoprotein IIb/IIIa inhibition improves endothelium-dependent vasodilatation in the forearm of patients with symptomatic coronary artery disease.[15]

The data from this study also showed that tirofiban reduced infarct size and improved ventricular function, which is in agreement with the reports of Kunichika *et al*,[8] Mak *et al*[10] and Danzi *et al*,[43] whereas the aspirin–clopidogrel combination did not. This is also the first study to show that antiplatelet treatment with tirofiban and aspirin–clopidogrel combination has totally different effects in reducing infarct size. Barrabes *et al*[44] reported, however, that the glycoprotein IIb/IIIa inhibitor lamifiban, administered intravenously 5 min before reperfusion, did not reduce infarct size after ischaemia and reperfusion. One possible explanation for the discrepancy may be the timing of administration. The mechanism of glycoprotein IIb/IIIa inhibition in reducing infarct size is not well defined. Kunichika *et al*[8] postulated that the favourable effects of tirofiban on infarct size might have been related to a rapid restoration of microvascular blood flow, thus reducing myocardial infarct size. The beneficial effect of tirofiban on ventricular function was due not only to decreased myocardial necrosis but also to preservation of microvascular integrity and improved myocardial tissue perfusion during AMI and reperfusion.

Study limitations

This study has several limitations. The dose of tirofiban used may be low in the mini-swine model. In fact, it has been shown recently that with regimens used in the RESTORE study (10 μg/kg bolus, followed by 0.15 μg/kg/min infusion), the average extent of inhibition of platelet aggregation was suboptimal (only 60–66% of baseline values)[45] and that glycoprotein IIb/IIIa inhibitor increased the inhibitory effects in a dose-dependent manner.[46] Hence we may benefit from a dose-ranking investigation obtained by modulating the dose of tirofiban later. Heparin or a lytic drug was not administered in this model and hence the contribution of glycoprotein IIb/IIIa inhibition in combination with these agents was not assessed. Because we assessed infarct size at 1 h of reperfusion, the ultimate infarct size may be larger. Although we started the tirofiban infusion before rather than after occlusion of the infarct vessel, administration before reperfusion has already been shown to be effective.[8–10] Lastly, the results were observed in a short-term experimental setting, and no long-term data are available.

In conclusion, the present study showed that tirofiban was very effective in attenuating myocardial no-reflow and endothelial function after reperfusion; in contrast, aspirin and clopidogrel in combination was totally ineffective. These findings suggest that tirofiban reduces myocardial no-reflow mainly by endothelial protection rather than by platelet inhibition.

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Supported in part by a grant-in-aid (90209038) from the National Natural Science Foundation of China.

Competing interests: None declared.

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